

Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions

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The Polycomb group (PcG) proteins form chromatin-modifying complexes that are essential for embryonic development and stem cell renewal and are commonly deregulated in cancer. Here, we identify their target genes using genome-wide location analysis in human embryonic fibroblasts. We find that Polycomb-Repressive Complex 1 (PRC1), PRC2, and tri-methylated histone H3K27 co-occupy >1000 silenced genes with a strong functional bias for embryonic development and cell fate decisions. We functionally identify 40 genes derepressed in human embryonic fibroblasts depleted of the PRC2 components (EZH2, EED, SUZ12) and the PRC1 component, BMI-1. Interestingly, several markers of osteogenesis, adipogenesis, and chondrogenesis are among these genes, consistent with the mesenchymal origin of fibroblasts. Using a neuronal model of differentiation, we delineate two different mechanisms for regulating PcG target genes. For genes activated during differentiation, PcGs are displaced. However, for genes repressed during differentiation, we paradoxically find that they are already bound by the PcGs in nondifferentiated cells despite being actively transcribed. Our results are consistent with the hypothesis that PcGs are part of a preprogrammed memory system established during embryogenesis marking certain key genes for repressive signals during subsequent developmental and differentiation processes.

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The human body consists of at least 200 different types of cells. The response of each of these cells to extra- or intracellular signals depends on their particular lineage "identity." In other words, despite having identical genomes, cells can respond in markedly different ways to the same stimulus. Therefore, a strong "programming" system must function to preserve and specify the identity of each cell type. It has become increasingly clear in recent years that maintenance of cell identity and, to a certain extent, also specification of cell identity are controlled by epigenetic events (Fisher 2002; Jaenisch and Bird 2003; Hsieh and Gage 2004; Valk-Lingbeek et al. 2004). These epigenetic events, which include DNA methylation and post-translational modifications of the histones, control the transcriptional program of each cell by regulating chromatin structure. The histone modifications are thought to constitute an indexing mechanism, referred to as the "histone code" (Jenuwein and Allis 2001), containing information such as the past and

present gene activity and the physiological status of the cell. This code is deciphered by other protein complexes, which specifically recognize and bind the modified histones and, in turn, elicit the biological effects of the imprints. The Polycomb repressors and Trithorax activators are thought to be the central players in these epigenetic programming events (Orlando 2003; Molofsky et al. 2004; Valk-Lingbeek et al. 2004).

Polycomb group genes (PcGs) are usually considered as being transcriptional repressors that are required for maintaining the correct spatial and temporal expression of homeotic genes during development and were originally identified based on studies demonstrating that deletions of PcG genes lead to homeotic transformations of fruit flies (Orlando 2003; Ringrose and Paro 2004; Pirrotta and Gross 2005). The vertebrate homeotic *HOX* genes are located in four distinct clusters (A, B, C, D) that are organized into 13 homology (or paralog) groups (Pearson et al. 2005). The chromosomal organization of the genes in each *HOX* cluster reflects its anterior-posterior expression in the body plan. The "tail" *HOX* genes at the 5' end of the *HOXA* locus (i.e., *HOXA7-13*) are expressed predominantly in the posterior of the developing embryo. In PcG knockout mice, the repression of these

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"tail" *HOX* genes is impaired in the middle and head part of the embryo, resulting in posterior-to-anterior transformation defects (Levine et al. 2004; Lund and van Lohuizen 2004).

Recent biochemical approaches have established that the PcG proteins form multiprotein complexes, called Polycomb-Repressive Complexes (PRCs). PRC2 contains EZH2, EED, SUZ12, and RbAp48, while the PRC1 complex consists of >10 subunits including the oncoprotein BMI-1 and the HPC proteins (CBX2, CBX4, CBX7, CBX8), HPH1-3, RING1-2, and SCML (Levine et al. 2004; N. Dietrich, K. Helin, and K.H. Hansen, unpubl.). Functionally, EZH2 is the catalytically active component of PRC2, acting as a histone methyltransferase specific for Lys 27 (K27) of histone H3 and K26 of histone H1 (Cao and Zhang 2004; Kuzmichev et al. 2004). Interestingly, the HPC proteins of the PRC1 complex can specifically bind to tri-methylated H3K27 (H3K27me3) (Cao and Zhang 2004; Kuzmichev et al. 2004; N. Dietrich, K. Helin, and K.H. Hansen, unpubl.). Since PRC2 is required for PRC1 binding to chromatin (Rastelli et al. 1993; Hernandez-Munoz et al. 2005), it has been proposed that this is primarily achieved through binding of HPC proteins to H3K27me3. Recently, the PRC1 complex has been demonstrated to possess a H2A-K119 ubiquitin E3 ligase activity that, like H3K27me3 activity, is associated with the repression of *HOX* genes (Cao et al. 2005).

In addition to being essential regulators of embryonic development, the PcGs have also emerged as key players in the maintenance of the adult stem cell populations (Molofsky et al. 2004; Valk-Lingbeek et al. 2004). For example, BMI-1 is required for the self-renewal of hematopoietic and neural stem cells (Lessard and Sauvageau 2003; Molofsky et al. 2003), while overexpression of EZH2 is capable of blocking the differentiation of muscle myoblasts (Caretta et al. 2004) and preventing hematopoietic stem cell exhaustion (Kamminga et al. 2005). Consistent with their critical roles in development, differentiation, and stem cell renewal, several PcGs are oncogenes, overexpressed in both solid and hematopoietic cancers (Pasini et al. 2004a; Valk-Lingbeek et al. 2004; Raaphorst 2005).

Although substantial progress has been made toward understanding the biological and biochemical functions of PcG proteins, we still know little about how precisely they control development and cell fate decisions. Since it is believed that they primarily function as epigenetic regulators of transcription, we performed chromatin immunoprecipitations (ChIP) and genome-wide screenings using tiled arrays to identify PcG target genes in human cells. Strikingly, we observe a very strong bias for genes controlling development and cell fate decisions. We investigate the functional relevance of PcG regulation in both human embryonic fibroblasts and in a model of neuronal differentiation. Moreover, we show that the PcGs target several tumor suppressor genes and genes known to be down-regulated in cancer. Based on these data, we propose models for how the PcGs control transcription.

Results

Identification of gene expression changes in cells depleted of PRC1 and PRC2 members

To obtain an understanding of how the PcGs control development and cell fate decisions, we chose to identify their target genes. To do this, we wanted to perform ChIP experiments using specific PcG antibodies followed by probing of microarrays (chips) with the enriched material (ChIP-on-chip). As an initial screen, we identified candidate genes by performing expression array analysis of PcG-depleted cells. Then subsequently, by generating tiled arrays mapping the complete loci of these potential target genes and probing with the enriched material, we aimed to identify functionally relevant direct targets.

We transfected proliferating human embryonic diploid fibroblasts (TIG3) with small interfering RNA (siRNA) oligonucleotides specific for the PRC2 components (EZH2, EED, and SUZ12) or the PRC1 component BMI-1. Western blot analysis of lysates prepared 44 h after transfection confirmed that the siRNAs efficiently inhibited the protein synthesis of their specific targets (Fig. 1A). As described previously, the protein levels of PRC2 members are in part dependent on the presence of the other partners in the complex (Pasini et al. 2004b). RNA was extracted at this point and labeled and hybridized to Affymetrix gene expression arrays (HG-U133). The short incubation period with siRNA oligonucleotides generated small but reproducible expression changes in several hundred genes. We observed a substantial overlap in gene expression changes after inhibiting the expression of BMI-1 and the three proteins of the PRC2 complex (Fig. 1B). In order not to miss any relevant genes for the subsequent ChIP-on-chip analysis, we selected a total of 341 genes as potential PcG target genes, whose expression is significantly changed upon down-regulation of at least three out of the four PcG proteins analyzed in our experiments (Fig. 1C; Supplementary Table 1). The reliability of the expression array data was confirmed by performing quantitative real-time PCR analysis (qPCR) of a selection of 18 genes (Fig. 1D; data not shown).

PcG binding and overlapping H3K27 tri-methylation of complete gene loci in the HOX gene clusters

To identify target genes by ChIP analysis, we first screened several candidate antibodies specific for PRC1 and PRC2 proteins and the tri-methylated H3K27 for their ability to efficiently coimmunoprecipitate histone H3 in ChIP conditions (data not shown). From this analysis we selected antibodies specific for SUZ12 (PRC2), CBX8 (PRC1), and H3K27me3. To validate the specificity of these antibodies in the ChIP-on-chip analysis, we represented the entire *HOXA*, *HOXB*, *HOXC*, and *HOXD* loci on tiled chips. We probed these chips with DNA amplified by ligation-mediated PCR from ChIPs performed with antibodies specific for H3K27me3, SUZ12, and CBX8 using an antibody for HA as a negative

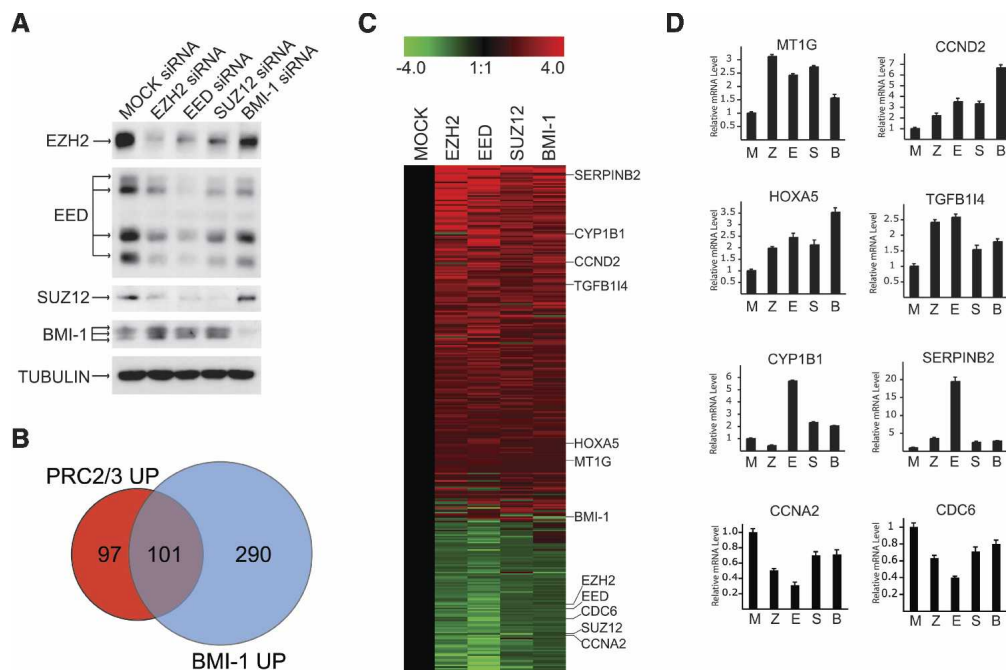


Figure 1. Genome-wide expression screen to identify gene changes in Polycomb depleted cells. (A) Western blot analysis of lysates prepared from TIG3 fibroblasts 44 h after transfection with siRNA oligos designed to inhibit the expression of EZH2, EED, SUZ12, or BMI-1. Tubulin was used as a loading control. (B) Venn diagram depicting the overlap in genes increased >1.2-fold in PRC2 (EZH2, EED, and SUZ12 combined) and PRC1 (BMI-1) depletion. (C) Treeview representation of Affymetrix expression data depicting the gene expression changes in Polycomb-depleted cells. (D) Validation of gene expression changes by qPCR for a selection of genes (indicated in B). mRNA was prepared from TIG3 cells transfected with Mock (M), EZH2 (Z), EED (E), SUZ12 (S), or BMI-1 (B) siRNAs. The experiments were performed independently of the gene expression array experiments.

control and an antibody for E2F3 as a positive control for the ChIP technique. In Figure 2, A and B, the enrichments observed across the entire *HOXA* locus are depicted at high resolution in Log₂ and normal scale, respectively. Very strong enrichments of SUZ12, CBX8, and H3K27me3 were observed immediately downstream of the *HOXA9* gene and extend upstream of the *HOXA13* gene, a stretch of ~45 kb. Interestingly, the enrichments observed on the *HOXA9–13* genes “blanket” their complete gene loci, and are not restricted to their promoter regions. Similar robust enrichments were observed on the *HOXB*, *HOXC*, and *HOXD* loci (Supplementary Fig. S1). As a further confirmation of the antibody specificity, we did not observe any significant enrichment of PcGs or H3K27me3 on a large number of control genes, including the E2F target genes *CDC6* and *CCNA2* (Supplementary Fig. S2).

To validate the enrichment profiles observed on the tiled chips, we quantified the immunoprecipitated DNA enrichments by qPCR of an independent ChIP experiment using primers designed within the promoter regions of all the *HOXA* genes (Fig. 2C). This established that EZH2 binds along the *HOXA* locus with an almost identical profile to SUZ12, CBX8, and H3K27me3. Significantly, Polymerase II was only detected at the promoters of the *HOXA4* and *HOXA5* genes, which are the only paralogs expressed in embryonic fibroblasts, consistent with the fact that these cells are of mesodermal origin.

Identification of target genes whose expression is regulated by the PcGs

Next, we represented the 341 gene loci of the genes identified in the expression array analysis starting from 15 kb upstream of the transcriptional start site (TSS) and ending 5 kb downstream of the 3' end of the last known exon (Supplementary Table 1) on custom designed tiled chips. We hybridized these chips with the DNA used for the analysis of the *HOX* loci. Specific enrichments of CBX8, SUZ12, and H3K27me3 were detected either at the promoter or elsewhere within the gene locus of 43 genes, represented as a treeview plot in Figure 3A. A detailed description of the exact PcG-binding positions on these genes is given in Supplementary Table 2. We observed enrichments of two types, either in confined “bell curves” that center around a particular point or as the “blanket” type, possibly consisting of multiple peaks fused together. For example, SUZ12, H3K27me3, and CBX8 form bell curve enrichments within the TSS of the *ATF3*, *BMP2*, and *DDK2* genes (Fig. 3B; Supplementary Fig. S3), while blanket-type enrichments are observed on the *OTX2*, *CCND2*, and *MT1G* genes (Fig. 3B; Supplementary Fig. S3; Supplementary Table 2). The *MT1G* gene is part of the metallothionein multigene cluster (*MT1E*, *MT1J*, *MT1A*, *MT1B*, *MT1F*, *MT1G*, and *MT1H*), which has blanket-type enrichments analogous to the *HOX* loci (Supplementary Fig. S3; Supplementary Table 3).

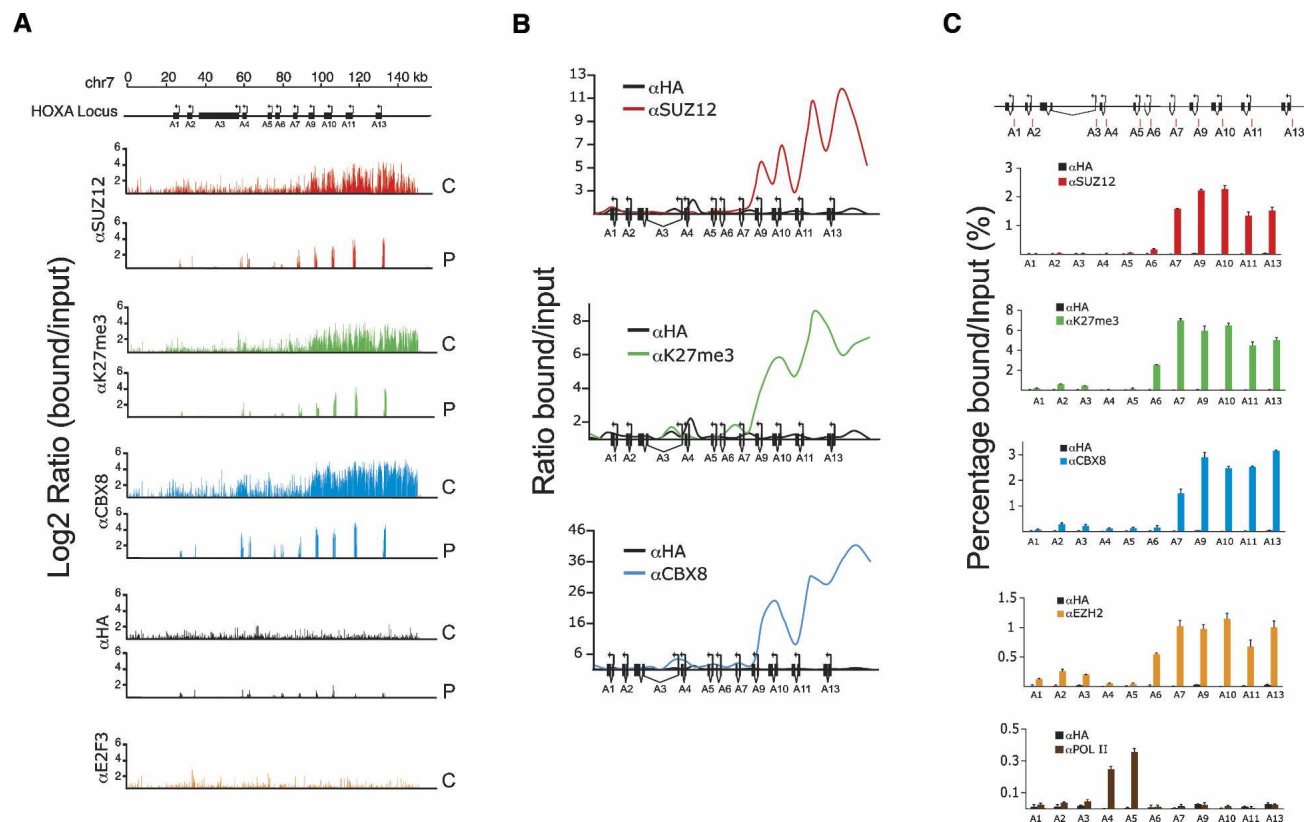


Figure 2. The PcG proteins and H3K27me3 are highly enriched on the *HOXA* gene cluster. (A) ChIP-on-chip tiling array analysis of the *HOXA* locus spanning 140 kb of DNA starting at position 26,880,000 on chromosome 7. High-resolution mapping of the 140 kb was achieved by representing 2840 probes of 50 bp with an average spacing of 3 bp between probes. The Y-axis represents the Log₂ signal ratios (bound/input) for the indicated antibodies. The results of the promoter ChIP-on-chip experiment are also shown for the *HOXA* promoters. In this analysis, chips were used containing 24,275 human promoters from 1300 bp upstream to 200 bp downstream of TSS with 15 50-bp probes with an average spacing of 100 bp between probes. (C) Custom ChIP-on-chip data; (P) Promoter ChIP-on-chip data. (B) XY scatterplot representation of the data depicted in A, created by plotting the average enrichments at 5-kb intervals. (C) Normal ChIP analysis of the promoters of the *HOXA* gene cluster. Primers were designed within the promoter regions (indicated with red bars) of all *HOXA* genes as indicated at the top of the panel. Enrichment is shown as percentage input.

Intriguingly, SUZ12 and CBX8 are also found on the *BMI-1* and *CBX8* gene loci and are associated with H3K27me3 enrichments (Fig. 3B; Supplementary Fig. S3). The expression of *BMI-1* and *CBX8* increases following the siRNA-mediated depletion of other PRC1 or PRC2 members (Figs. 1A, 3A). Therefore, these data suggest that PcG proteins autoregulate their own synthesis. This is conserved during evolution, since PcG proteins bind to the polyhomeotic (Ph) locus in *Drosophila* (Fauvarque et al. 1995; Bloyer et al. 2003).

Next we validated a selection of these newly identified target genes, for EZH2, SUZ12, CBX8, and H3K27me3 enrichments in an independent experiment (Fig. 3C). The previously identified SUZ12 target gene *MYT1* (Kirmizis et al. 2004) was included as a positive control and the *HOXA1* and *CCNA2* genes as negative controls. Finally, the expression of several target genes (*MT1G*, *CCND2*, *SERPINB2*, and *CYPB1*) were tested and shown to be reduced in *BMI-1*-overexpressing TIG3 cells (data not shown), further validating them as PcG target genes in this cellular system. We conclude that we have iden-

tified 43 target genes of the PcG proteins, whose expression changes in PcG-depleted cells.

Perhaps surprisingly, the mRNA transcripts of the previously characterized SUZ12 target gene *MYT1* as well the *HOXA7-13* genes remained undetectable in PcG-depleted cells (data not shown). This is despite the fact that we observe strong PcG enrichments on their promoters (Figs. 2C, 3C). Therefore, in order to identify all PcG target genes in human embryonic fibroblasts, we decided to perform a global unbiased identification of PcG-bound promoters independent of their expression.

Genome-wide identification of human promoters bound by PcG

We interrogated chips containing probes for 24,275 human promoters within a defined region spanning 1300 base pairs (bp) upstream to 200 bp downstream of the TSS. Significantly, the PcGs bind within this limited promoter region in 70% of the target genes described in Figure 3A (see Supplementary Table 2). As shown in Fig-

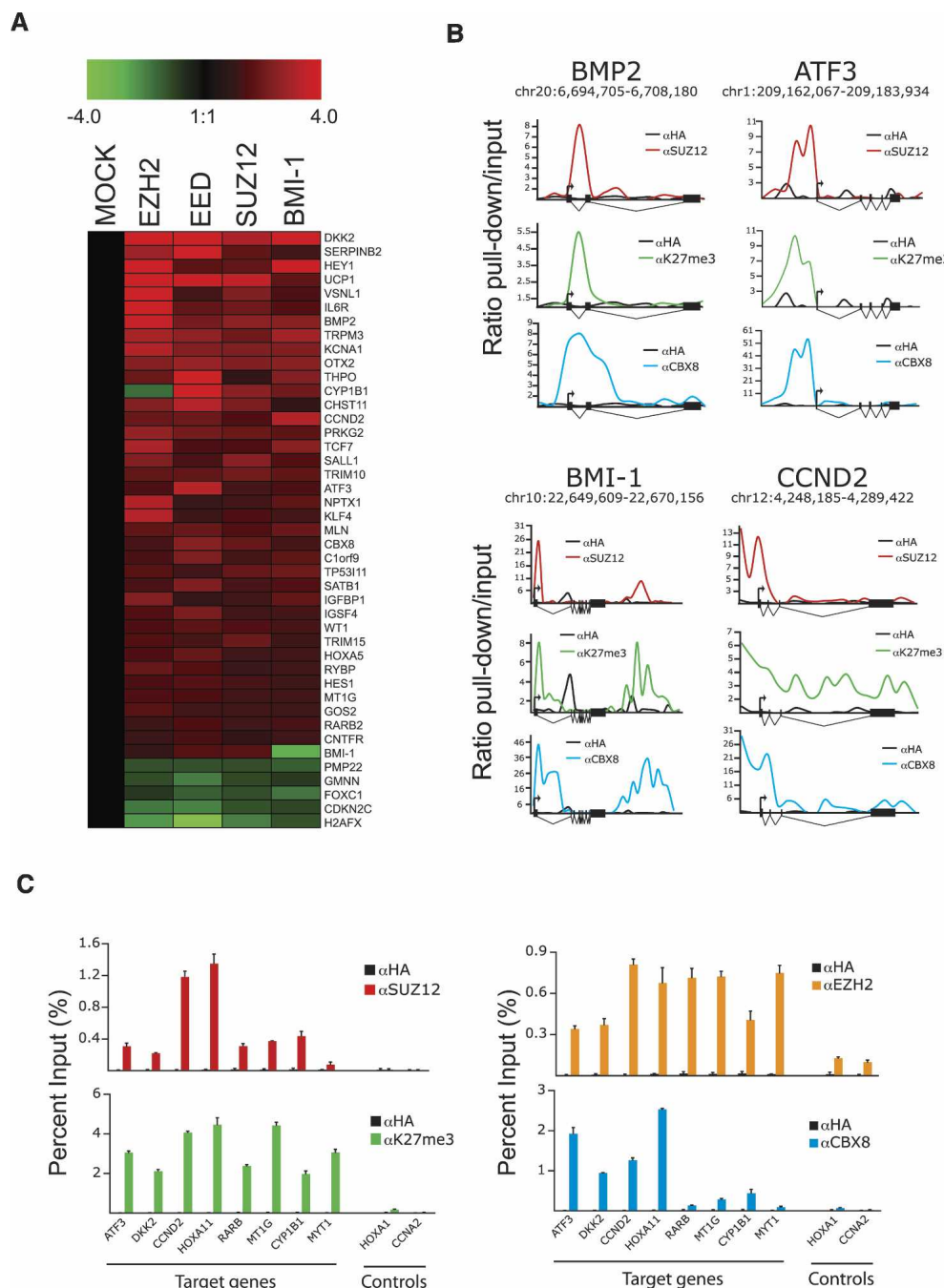


Figure 3. Identification of 43 PcG target genes whose expression changes in Polycomb-depleted cells. (A) Treeview depiction of the expression changes in Polycomb-depleted cells of 43 direct PcG target genes. (B) XY scatterplot representations of SUZ12, H3K27me3, and CBX8 enrichments along the gene loci of *BMP2*, *ATF3*, *BMI-1*, and *CCND2*. The chromosome number and the region covered are depicted above each panel. (C) Standard ChIP analysis of newly identified Polycomb target genes using the previously identified *MYT1* target gene as a positive control. The *CCNA2* and *HOXA1* genes are presented as negative controls.

ure 4A and listed in Supplementary Table 3, SUZ12, CBX8, and H3K27me3 are present on a very large number of promoters. A significant enrichment was found for CBX8 (PRC1) on 2487 promoters (10.2% of the total), for H3K27me3 on 2206 promoters (9.0%), and for SUZ12 (PRC2) on 1042 promoters (4.3%). A very significant overlap was observed between promoters bound by

PRC1 and PRC2, which were also tri-methylated on H3K27, consistent with the fact that PRC1 is dependent on PRC2-mediated K27 tri-methylation for its ability to bind to chromatin (Hernandez-Munoz et al. 2005; Pirrotta and Gross 2005). Several genes with H3K27me3 enrichments appeared not to have associated CBX8, suggesting that this mark may execute distinct functions

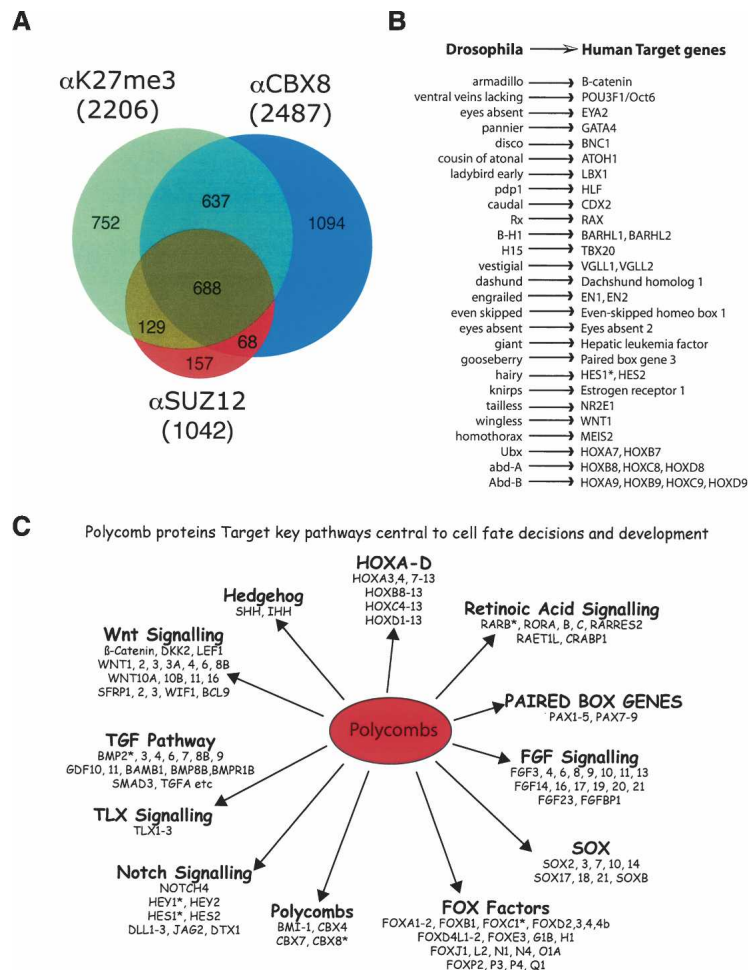


Figure 4. Genome-wide mapping of PcG target promoters. (A) Venn diagram depicts significant overlaps between the presence of SUZ12, CBX8, and H3K27me3 on promoters. (B) A remarkable conservation of Polycomb target genes through evolution from *Drosophila* to humans. Several confirmed or predicted *Drosophila* PcG target genes are shown in the left column. In our analysis we have identified their human homologs as being human PcG targets (see Supplementary Table 3). (C) A selection of PcG target genes identified in this study, focusing on those known to be involved in key pathways controlling development, differentiation, stem cell biology, and cell fate decisions.

independent of PRC1 complexes. Alternatively, PRC1 might be bound to these genes through the two chromo-domain-containing CBX8 homologs CBX4 or CBX7. The large number of genes identified, which were only significantly bound by CBX8, are unlikely to be CBX8 specific targets. In fact, genes belonging to this category, when subsequently tested in quantitative ChIP analysis, were found to also possess significant H3K27me3 and SUZ12 binding, for example, *CYP1B1* and *DDK2* [cf. Fig. 3C and Supplementary Table 3]. Therefore the large number of genes bound only by CBX8 is likely a consequence of the higher affinity of the CBX8 antibody for its epitope rather than a reflection of PRC2-independent recruitment. In conclusion, our results using promoter arrays extend the repertoire of PcG target genes to >1000 (Fig. 4A; Supplementary Table 3).

Next we asked: What is the nature of the target genes identified? Remarkably, we found that the majority of the genes previously identified or predicted to be PcG target genes in *Drosophila* have human homologs identified as PcG targets in this screen (Fig. 4B; Ringrose and Paro 2004; Ringrose et al. 2004). In addition to the *HOX* genes, we found that *Engrailed*, *Hedgehog*, *Hairy*, and *Caudal* all have human PcG target homologs. Interestingly, another target in *Drosophila* is the Polyhomeotic

locus. As mentioned above, we have found that the human PcGs also bind to the *BMI-1* and *CBX8* gene loci, and now extend this group to include the *CBX4* and *CBX7* loci.

PcGs target both stem cell- and differentiation-specific genes

We wished to study PcG dynamics on target genes in a biologically relevant model of differentiation. The global mapping analysis revealed that the PcGs bind many genes that are specifically induced in various types of differentiation (Table 1). For example, *BMP6* (Kugimiya et al. 2005), the homeobox gene *PAX4* (Wang et al. 2004), *MYOG* (Rohwedel et al. 1994), *DMBT1* (Al-Awqati 2003), and *ZIC1* (Sato et al. 2005) are specific differentiation factors induced in osteogenesis, endocrinal differentiation, myogenesis, epithelial cell differentiation, and neurogenesis, respectively. Recent results have shown that EZH2 represses the muscle creatine kinase gene *CKM* in nondifferentiated myoblasts and is displaced from the gene promoter upon muscle differentiation, when the gene becomes activated (Caretta et al. 2004). Our results show that the PcGs target a large number of tissue-specific differentiation genes like *CKM* (see Table

Table 1. *Examples of differentiation genes identified by PcG ChIP-on-chip*

Gene name	Function	Antibodies used			
		α HA	α CBX8	α SUZ12	α K27me3
DLX5	Neuronal differentiation		+	+	+
HEY2	Neuronal differentiation		+	+	+
LHX1	Neuronal differentiation		+	+	+
MASS1	Neuronal differentiation		+	+	+
NEFL	Neuronal differentiation		+	+	+
NEUROD1	Neuronal differentiation		+	+	+
NEUROG1	Neuronal differentiation		+	+	+
NEUROG2	Neuronal differentiation		+	+	+
NMU	Neuronal differentiation		+	+	+
NXPH2	Neuronal differentiation		+	+	+
OLIG2	Neuronal differentiation		+	+	+
PAX3	Neuronal differentiation		+	+	+
PHOX2A	Neuronal differentiation		+	+	+
PHOX2B	Neuronal differentiation		+	+	+
POMC	Neuronal differentiation		+	+	+
POU4F2	Neuronal differentiation		+	+	+
ROBO3	Neuronal differentiation		+	+	+
SEMA5B	Neuronal differentiation		+	+	+
SIM1	Neuronal differentiation		+	+	+
TBR1	Neuronal differentiation		+	+	+
ZIC1	Neuronal differentiation		+	+	+
BMP3	Bone differentiation		+		
TNFSF11	Bone differentiation		+		+
CHRD2	Bone differentiation		+	+	+
BMP6	Bone differentiation		+		
GDF2	Bone differentiation		+		+
BMP7	Sex differentiation			+	+
JAG2	Sex differentiation				+
DMRT1	Sex differentiation		+	+	+
DMRT2	Sex differentiation		+	+	+
DMRT3	Sex differentiation		+	+	+
SRX	Sex differentiation		+		
DMRTA1	Sex differentiation		+		+
BMP8B	Sex differentiation		+		+
FGF9	Sex differentiation		+		
CD4	T-cell differentiation				+
MDFI	Chondrogenic differentiation		+	+	+
CSRP3	Muscle development		+		
MYOG	Muscle development		+		+
SYNE1	Muscle development		+		
CKM	Muscle development			+	+
MYH11	Muscle development		+	+	+
ACHE	Muscle development		+	+	+
TNNT1	Muscle development		+	+	+
HAND2	Cardiogenesis		+		
DLL1	Cell fate/Notch pathway		+		
DLL3	Cell fate/Notch pathway		+	+	+
NOTCH4	Cell fate/Notch pathway		+		+
PAX4	Endocrine differentiation		+	+	+
EDAR	Epidermal cell differentiation		+		+
DMBT1	Epithelial cell differentiation		+		+
THPO	Hematopoiesis		+	+	+
KIT	Hematopoiesis		+	+	+
LIF	Hematopoiesis		+		
KDR	Hematopoiesis		+		+
TAL1	Hematopoiesis	+	+	+	+
BMP4	Mesoderm cell fate decisions		+		
SHH	Mesoderm cell fate decisions		+	+	+

A selection of enriched target genes identified in the ChIP-on-chip analysis using a 24,275 tiled promoter array.

1), suggesting that the displacement of PcGs from differentiation-specific genes during terminal differentiation is a general phenomenon (depicted in Fig. 5A, panel i).

To test this, we chose to focus on neuronal differentiation for several reasons. A large number of neuronal differentiation genes are bound by PcGs (Table 1). The PcGs are required for neuronal development and self-renewal of neural stem cells (Leung et al. 2004; Molofsky et al. 2004). Additionally, there are several well-established model systems for neuronal differentiation processes. As a model system, we chose the human embryonic teratocarcinoma cell line NT2/D1, which has neural progenitor cell properties and irreversibly differentiates along a neural lineage upon treatment with retinoic acid (RA) (Lee and Andrews 1986).

We selected four genes based on their proposed role in neuronal differentiation: the gene for the zinc-finger domain transcription factor *ZIC1* required for normal neuronal differentiation (Grinberg and Millen 2005); the homeobox-containing transcription factor *MEIS2*; the RA receptor β , *RARB*; and the gene for neurofilament light chain, *NEFL* (Zhu et al. 1997). The expression of these genes is induced during differentiation (Zhu et al. 1997; Niederreither et al. 2000) and during RA-induced differentiation of NT2/D1 cells (Fig. 5B; data not shown). Consistent with the increased expression of these genes during differentiation, we observed a progressive decrease in PcG binding and H3K27me3 enrichments on these genes (Fig. 5B; data not shown). These data are consistent with the model depicted in Figure 5A, panel i, and since the PcGs associate with a large number of tissue-specific genes in differentiated cells, this suggests that the differentiation specific signals, which activate tissue-specific genes, lead to the displacement of PcGs from the genes.

Subsequently we decided to investigate PcG target genes, which become silenced during differentiation. A potential model for how this would occur is depicted in Figure 5A, panel ii, in which we speculate that PcGs would be recruited during differentiation. To test this hypothesis, we selected three genes that are highly expressed in neuronal progenitor cells but silenced during differentiation (Fig. 5C). These included genes for the basic helix-loop-helix (bHLH) proneural transcription factors *OLIG2* (Lee et al. 2005) and *NEUROG2* (Ma et al. 1996), and the gene for metallothionein 1G, *MT1G*. ChIP experiments showed, in contrast to our expectations, a strong and significant binding of PcGs together with the presence of H3K27me3 on the promoters of these genes in undifferentiated cells (Fig. 5C), with only slight increases during differentiation. These results demonstrate that binding of PcGs to target genes in undifferentiated cells does not strictly correlate with transcriptional silencing.

To understand if this intriguing result could be extended to other genes, we analyzed the binding of the PcGs to the *HOXA* locus before and after differentiation of the NT2/D1 cells. Previous data have shown that the *HOX* genes display faithful regulation patterns following RA-induced differentiation of NT2/D1 cells (Simeone et al. 1990; Houldsworth et al. 2002). As depicted in Figure

6A and shown in Figure 6B, the (posterior) 5'-end genes (*HOXA7–13*) are expressed in undifferentiated cells but are dramatically repressed upon RA treatment. In contrast, the genes at the (anterior) 3' end of the locus (*HOXA1–5*) become strongly activated. We determined the binding of EZH2 and CBX8 together with H3K27me3 enrichments on the complete *HOXA* locus both before and after induction to differentiate. Consistent with the genes analyzed in Figure 5, the PcGs are displaced from the activated *HOXA1–5* gene loci during differentiation, while they are already bound to the *HOXA7–13* genes in undifferentiated NT2/D1 cells (Fig. 6C) and remain bound throughout the differentiation process and subsequent decrease in mRNA expression. These results on the *HOXA* cluster expand the set of genes, which are transcriptionally active in undifferentiated cells yet bound by PcGs prior to their repression in differentiated cells.

Discussion

By genome-wide location analysis, we have identified >1000 putative genes bound by PcG proteins. Strikingly, these genes contain the key members of the Wnt, TGF β , FGF, Notch, and Hedgehog signaling pathways known to be the regulators of developmental and differentiation processes. In addition, target genes are conserved throughout evolution from *Drosophila* to human, suggesting that this transcriptional regulatory network is essential for the development and differentiation of all multicellular organisms.

Polycomb target genes

By performing gene expression profiling of human embryonic fibroblasts in combination with ChIP-on-chip experiments, we identified 43 PcG target genes whose expression is dependent on the PcGs. The large majority of these genes become derepressed as a result of specific depletion by siRNAs of the PRC2 components (EZH2, EED, and SUZ12) or the PRC1 component BMI-1 (39 of 43). These target genes include several known markers of bone, cartilage, and fat differentiation (Supplementary Table 2). To put this into context, fibroblasts are proliferating cells of the mesoderm that compose part of the connective tissue in almost every tissue and organ. They are derived from mesenchymal stem cells, which are multipotent precursors, capable of differentiating into osteoblasts, adipocytes, chondrocytes, endothelial cells, and also non-mesoderm-type lineages, such as neuronal-like cells (Kassem 2004). Interestingly, evidence exists that embryonic fibroblasts in tissue culture maintain at least some of the multipotency of mesenchymal stem cells. For example, mouse embryonic fibroblasts can undergo chondrogenic and adipocytic differentiation (Ge et al. 2002; Lengner et al. 2004). Consistent with this, we found that several markers of osteocytic, chondrocytic, adipocytic, and neural differentiation are up-regulated in Polycomb-depleted cells (Fig. 3A; Supplementary Table

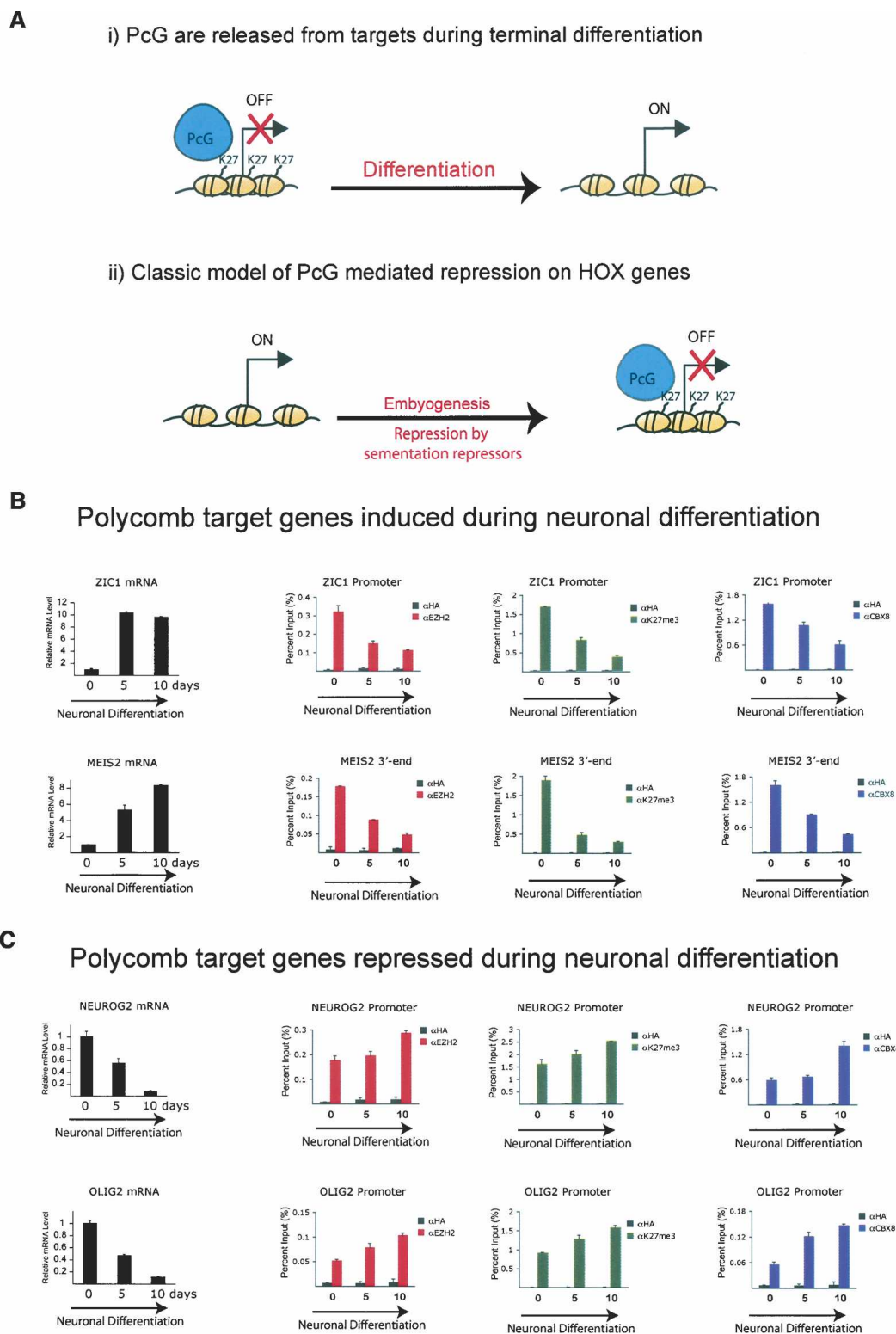


Figure 5. PcGs bind to genes that are repressed or induced during neuronal differentiation. (A) Models for how PcGs could regulate gene expression during terminal differentiation. (B) qPCR and ChIP analysis of two PcG target genes (*ZIC1* and *MEIS2*) induced during neuronal differentiation of NT2/D1 cells treated with 1 μ M RA. (C) qPCR and ChIP analysis of two genes (*NEUROG2* and *OLIG2*) repressed during neuronal differentiation of NT2/D1 cells treated with 1 μ M RA.

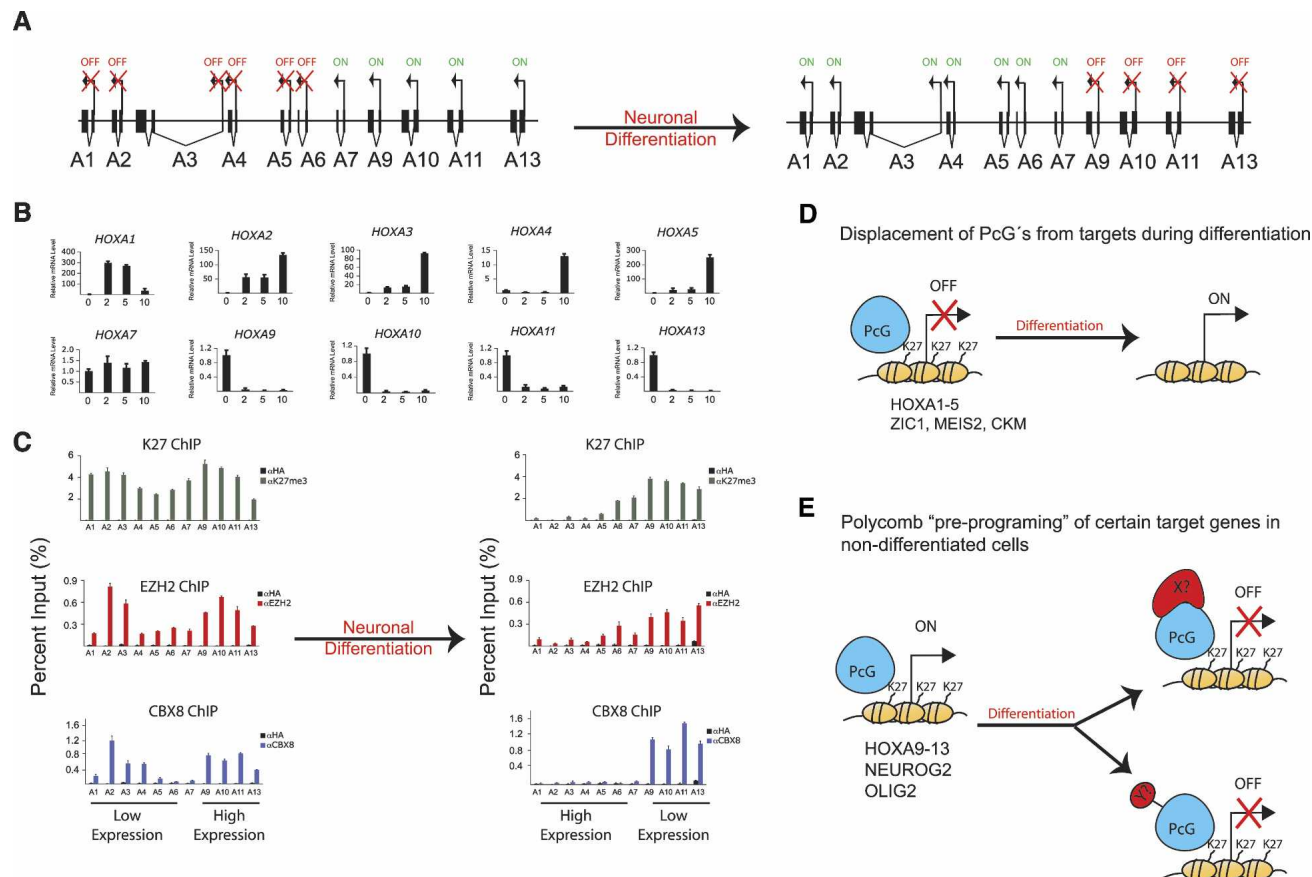


Figure 6. Polycombs are present on active *HOX* genes in nondifferentiated cells. (A) Schematic depiction of the gene expression changes along the *HOXA* locus upon RA-mediated induction of neuronal differentiation. (B) Quantification of mRNA expression changes of *HOXA* genes during neuronal differentiation of NT2/D1 cells by qPCR. (C) ChIP analysis of EZH2, H3K27me3, and CBX8 binding to the promoters of the *HOXA1* to *HOXA13* genes both before and after 10 d of RA treatment. (D) Displacement of PcGs from target genes activated during differentiation, for example, *ZIC1*, *MEIS2*, *RARB*, and *HOXA1-5* in neuronal differentiation and *CKM* in myoblast differentiation. (E) Polycomb "preprogramming" of certain target genes in undifferentiated cells, for example, *NEUROG2*, *OLIG2*, and *HOXA9-13*. These genes are paradoxically expressed while being bound by PcGs in undifferentiated cells. Several hypothetical mechanisms for the triggering of PcG repressive function during differentiation are possible, include post-translational modifications of the PcGs, addition of H1K26me3 or ubiquitinated H2A-K119 marks, recruitment of DNA methyltransferases, the binding of additional transcriptional repressors, or a combination of these mechanisms.

2). For example, both the bone morphogenic protein BMP2 and the Wnt/ β -catenin signaling protein *DKK2* function in terminal osteoblast differentiation (Li et al. 2005). While *HEY1* and *TCF7* are transcriptionally activated during osteogenesis (de Jong et al. 2004), both *CHST11* (Kluppel et al. 2005) and *PRKG2* (Chikuda et al. 2004) are associated with chondrocytic differentiation, the latter being a molecular switch from proliferation to hypertrophic differentiation of chondrocytes. The G0S2 protein is involved in adipocyte differentiation (Zandbergen et al. 2005), and UCP1 is a specific marker of Brown adipose tissue. In addition, several other target genes are known regulators of development such as *RARB*, *WT1*, and the homeobox genes *SALL1* and *HOXA5*. In summary, the combination of ChIP-on-chip and expression array analysis in human embryonic fibroblasts has identified 43 novel PcG target genes with important regulatory roles in mesenchymal differentiation and development.

Strikingly, the expression of only a relatively small number of the >1000 identified PcG target genes was affected by depleting PcGs in human embryonic fibroblasts, and >90% of the identified target genes are not detectably expressed. This is consistent with previous observations by Kirmizis et al. (2004) in SW480 colon cancer cells depleted of SUZ12. We hypothesize that the majority of the 1000 PcG target genes identified here in fibroblasts are permanently silenced. This permanent repression could be due to secondary epigenetic modifications of the target promoters by, for example, the EZH2 complex itself or by DNA methylation. Supporting this notion is the demonstration that EZH2 possesses H1K26 methylation activity (Kuzmichev et al. 2004), and since H1K26me3 can tether HP1 to chromatin (Daujat et al. 2005), this could add an additional layer of transcriptional repression on PcG target genes. Additionally, the expression of EZH2 has recently been shown to be sufficient for the recruitment of DNA methyl transferases

to cellular promoters leading to their DNA methylation and repression (Vire et al. 2005). It is possible that such secondary repressive modifications are not alleviated in PcG-depleted cells. As an alternative and complementary explanation for the lack of expression of a large number of PcG target genes in fibroblasts, we suggest that the fibroblasts may lack the specific transcriptional activators required for the expression of the tissue-specific target genes such as those listed in Table 1.

Polycomb target genes and cancer

The identification of PcG target genes may also provide vital mechanistic insights into the precise nature by which PcGs contribute to cancer. It has been proposed that cancer originates from nondifferentiated or stem cell-like cells, referred to as “cancer stem cells” (Reya et al. 2001). This idea is supported by the recent observation that mammary stem cells are enriched in premalignant breast tissue (Shackleton et al. 2006). The identification of a large number of PcG target genes required for differentiation (Table 1) strongly suggests that the oncogenic potential of genes such as BMI-1 and EZH2 can be ascribed to their role in stem cell maintenance. Another insight comes from the fact that a significant proportion of the identified PcG genes are silenced in cancer by DNA methylation of their promoter sequences. These include *RARB*, *CCND2*, *MT1G*, *KLF4*, *IGSF4*, *WT1*, *NPTX1*, *HOXA5*, *BMP2*, and *GOS2* (Evron et al. 2001; Loeb et al. 2001; Fackler et al. 2003; Fukami et al. 2003; Hagihara et al. 2004; Zhao et al. 2004; Henrique et al. 2005; Lewis et al. 2005). Significantly, as pointed out above, EZH2 has recently been shown to act as a platform for DNA methyltransferases on certain EZH2 target genes (Vire et al. 2005). The PRC2 complex could therefore contribute to cancer development by specifically silencing tumor-suppressor genes by DNA methylation. Consistent with this, we have found an inverse correlation between the expression levels of the PcGs, *EZH2*, *SUZ12*, and *BM1-1*, and the target genes *MT1G*, *HOXA5*, and *RARB* in breast cancer (A.P. Bracken, P. Cloos, and S. Confalonieri, unpubl.). It will be important in future investigations to determine if the silencing of these genes in cancer contributes to the development of the disease and if PcGs have a causal role in this.

Delineating the mechanisms by which PcGs regulate cell fate decisions

The identification of genes bound by PcGs has enabled us to begin to address the mechanisms by which they repress transcription. Our results, based on a model of neuronal differentiation, suggest at least two alternative mechanisms (depicted in Fig. 6D,E). The first model is exemplified by genes such as *ZIC1*, *MEIS2*, and *HOXA1-5*, which are bound and repressed by PcGs in undifferentiated cells. Upon induction to differentiate, PRC1 and PRC2 are displaced from these genes by, as yet, unidentified mechanism(s), leading to their derepression. This

model is consistent with previous results, suggesting a similar role for the PcGs in muscle and germ cell terminal differentiation processes (Caretta et al. 2004; Chen et al. 2005) and predicts that this is a general phenomenon.

The alternative model describes when PcG target genes are down-regulated during differentiation (Fig. 6E). This model is based on the surprising observation that the PcGs, in some cases, are bound to their target genes in undifferentiated cells, despite the gene being actively expressed. Based on our results for *OLIG2*, *NEUROG2*, and *HOXA9-13*, we propose that these PcG target genes are already “preprogrammed” to be repressed upon appropriate cell fate signals. Supporting this hypothesis, the Paro laboratory demonstrated that the *Drosophila* PcG and Trithorax proteins bind to Polycomb-Responsive Elements (PREs) and promoters of *HOX* genes before their expression levels are set by the early-acting segmentation factors (Orlando et al. 1998). Further supporting such a concept, we observed H3K27me3 and SUZ12 enrichments on the entire *HoxA* locus in mouse embryonic stem cells, resembling the profile observed in undifferentiated NT/D1 cells (Fig. 6C; Supplementary Fig. S4; data not shown). This suggests that the transcriptional memory system of the *HOX* genes and other genes controlling cell fate are preset or “programmed,” possibly by PcGs in the zygote generated from maternal mRNA. In this model, specific developmental signals such as RA concentrations could trigger the PcG-repressive capacity by as yet unidentified mechanisms. This could involve the post-translational modifications of the PcG proteins, the modification of other proteins/histones by the PcGs, or the specific recruitment/dissociation of transcriptional regulators from the PcG target genes (Fig. 6E). Therefore, we propose that the PRCs function as “platforms” for channeling repressive signals during developmental and differentiation processes.

In summary, we predict that PcGs form part of an epigenetic blueprint for development and differentiation established early in embryogenesis. Future investigations may hopefully unravel the precise mechanistic contributions of PcG proteins, modifications such as H1K26me3 and ubiquitinated H2A-K119 and associated proteins, such as HP1 and DNA methyltransferases, on the >1000 genes identified here during multiple types of cell fate decisions. An additional challenge for the future will be to harness this knowledge such that we can dedifferentiate or *trans*-differentiate cells for therapeutic purposes.

Materials and methods

Tissue culture

The human diploid embryonic lung fibroblast TIG3 cell line and the embryonic carcinoma cell line NTERA2 (NT2/D1) (Lee and Andrews 1986) were grown in DMEM supplemented with 10% (v/v) FCS. To induce differentiation of NT2/D1 cells, asynchronously growing cells were seeded at 30% confluency and were treated 24 h later with 1 μ M ATRA (dissolved in DMSO; Sigma). Cultures treated continuously with RA were collected at 0, 2, 5, and 10 d for RNA extraction or harvested for ChIP at 0, 5, and

10 d. Cultures were reseeded every 3–4 d and collected no less than 24 h after reseeding.

Generation of antibodies

Two polyclonal CBX8 (hPc3) antibodies “LAST” and “GALD” were produced in rabbits using synthetic peptides corresponding to amino acids 107–129 (KKPGRSPQDLASTSRAREGLRN MGL) and 295–318 (KKGQALDPNGTRVRHSGSPSSGG) of human CBX8, respectively. These were coupled to Keyhole Limpet Hemocyanin (KLH) through the N-terminal lysine residues and injected subcutaneously into rabbits according to standard procedures (DAKO). Positive sera produced were affinity-purified on the respective peptide antigens according to standard procedures. For ChIP assays, 5 µg of a pool of both affinity-purified antibodies was used per immunoprecipitation.

siRNA interference

Specific siRNA oligos targeting EZH2, EED, SUZ12, and BMI-1 mRNAs were described previously (Bracken et al. 2003; Pasini et al. 2004b). Cells were transfected using Oligofectamine (Invitrogen) and harvested 44 h after transfection.

Quantification of mRNA levels by qPCR

cDNA was generated by RT-PCR using the PE Applied Biosystems TaqMan Reverse Transcription Reagents. Reactions were determined using the SYBR Green I detection chemistry system (Applied Biosystems), using an ABI Prism 7300 Sequence Detection System. Ubiquitin was used as a control gene for normalization. The sequences of the primers used are available upon request.

Affymetrix expression analysis

Total RNA was extracted from mock, EZH2, EED, SUZ12, and BMI-1 siRNA-transfected cells. For each treatment, RNA was prepared from six independent experiments and was pooled into one sample to reduce the experimental variation. Targets for microarray hybridization were synthesized accordingly to the supplier's instructions (Affymetrix). The human GeneChip array U133A (Affymetrix), which interrogates ~33,000 transcripts, was used for gene expression profiling. Fifteen GeneChip arrays U133A were used for this analysis; for each different condition, three separate arrays were hybridized with pooled cRNA. Hybridization, washing, staining, scanning, and data analysis were performed at The Affymetrix Microarray Unit at the IFOM-IEO campus, Milan, Italy, according to the manufacturer's instructions. Expression levels were analyzed using Microarray Analysis Suite (MAS) 5.0 statistical algorithm software (Affymetrix), using the default parameters and scaling (TGT Value) signal intensities for all the GeneChip arrays to a value of 500. The mock siRNA treatment was used as a baseline condition for comparison with the polycomb siRNA-treated samples.

ChIP assays

ChIPs were performed and analyzed essentially as described previously (Bracken et al. 2003). The antibodies used were rabbit anti-E2F3 (sc-878; Santa Cruz), anti-PolII (sc-899; Santa Cruz), anti-H3K27me3 (Upstate), anti-SUZ12 (Upstate), anti-HA (sc-805; Santa Cruz), a mouse monoclonal specific for EZH2 (AC22) (Pasini et al. 2004b), and an equal mix of the polyclonal antibodies for CBX8, described above. For normal chromatin IPs, the immunoprecipitated DNA was quantified by real-time qPCR.

The sequences of the PCR primers are available upon request. Instead for the ChIP-chip analysis, the immunoprecipitated DNA was amplified by LMPCR as described previously (Ren et al. 2000) and hybridized to tiled arrays manufactured by NimbleGen Systems, Inc. (<http://www.nimblegen.com>).

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